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THE INTERNATIONAL ARABIC JOURNAL OF ANTIMICROBIAL AGENTS ISSN 2174-9094

2016
Vol. 6 No. 2:1
doi: 10.3823/786

Prevalence of *Legionella* among pneumonia patients and environmental water samples in an Egyptian University Hospital

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Abstract

Objectives: To diagnose *Legionella* infection and determine its incidence and risk factors in community-acquired pneumonia (CAP) patients hospitalized in the chest department and hospital-acquired pneumonia (HAP) patients admitted to the emergency and surgery ICU of Zagazig University Hospital. Additionally, the study determines the occurrence of *Legionella* genus in the water of this department.

Subjects and methods: One hundred clinically diagnosed pneumonic patients; 50 patients with CAP and 50 with HAP were the subjects of this laboratory-based, comparative cross sectional study. Full clinical history and lower respiratory tract specimens were collected from each patient. Water samples were taken from 25 water outlets. DNA was extracted by QIAamp DNA Mini Kit, and real time PCR amplification of 16s r-RNA gene was used for diagnosis of *Legionella* genus. Risk factors were analyzed by logistic regression analysis.

Results: *Legionella* genus was identified in 8 / 50 patients of CAP (16%), and 10/ 50 patients with HAP (20%). In CAP patients, the organism was prevalent in old- age, smoker males, with diabetes mellitus (DM) and/or chronic obstructive pulmonary disease (COPD). Gastrointestinal tract (GIT) and neurological manifestations were the main presentations. Seventy-five percent of these patients were admitted to ICU. For patients with HAP, hospitalization for more than ten days and having a stroke or head trauma were significant risk factors. Ten out of the 25 water samples tested were positive for *Legionella* genus; seven samples were from the chest department and three were from emergency ICU. No water-contamination with *Legionella* was found in the surgery ICU.

THE INTERNATIONAL ARABIC JOURNAL OF ANTIMICROBIAL AGENTS
ISSN 2174-9094
2016
Vol. 6 No. 2:1
doi: 10.3823/786

Conclusion: Diagnosis of *Legionella* should be considered for both CAP and HAP in our hospital. Periodic surveillance for detection of this organism with subsequent disinfection of water sources should be carried out.

Key-words: *Legionella*, Real time PCR, Water, Egyptian Patients

Introduction

Legionella is the causative agent of Legionnaires' disease (LD) and Pontiac fever [1]. Although most cases are caused by *L. pneumophila*, other species are also pathogenic [2-4]. *Legionella* bacteria are intracellular pathogens; antimicrobial agents that achieve intracellular concentrations higher than the minimal inhibitory concentration (MIC) are effective for their treatment, especially macrolides and quinolones [5]. The activities of levofloxacin and azithromycin are similar and both are considered the first-line therapy [6-7]. Patients with legionnaire disease who do not receive appropriate antibiotics can have bad prognosis and high mortality rate [8].

Legionellosis can be diagnosed with many modalities [9]. However, data from developing world are scarce [10]. Detection of *Legionella* in water sources could be used as a predicting risk factor for LD [11-12].

In Egypt, the magnitude of legionellosis is not well recognized. Additionally, the few previous studies carried out addressed only *L. Pneumophila* [13, 14]. In Zagazig university hospital, to our knowledge, there is no information either on the prevalence and risk factors, or on water contamination with the general genus of *Legionella*.

The objectives of this study were to determine the incidence of *Legionella* genus among patients with pneumonia; both in community- and hospital-acquired and to identify risk factors associated with this infection. Additionally to determine the occurrence of *Legionella* genus in the water sources.

Subjects and Methods

The study was conducted over the period from January 2013 to January 2015 in the Zagazig University Hospital; which has 1800 bed capacity. It is a university-affiliated hospital located in Sharkya Governorate / eastern province of Egypt, with a catchment area of about 7 millions. The study was conducted under the supervision of both microbiology and chest departments.

Subjects

One hundred diagnosed pneumonic patients were enrolled in this laboratory-based, comparative cross sectional study. Fifty patients were diagnosed as CAP because they were admitted to the chest department with pneumonia. HAP cases were 50 patients who acquired pneumonia after a minimum of 7 days after admission to emergency ICU or surgery ICU and were diagnosed to have nosocomial pneumonia [15]. Complete medical history and clinical data were collected from all patients using a pre- prepared sheet.

Inclusion criteria were the presence of at least one of the major criteria for diagnosis of pneumonia, and these include; cough, sputum production, temperature >37.8°C or presence of at least two

Received on 14 May 2016, Accepted on 2 June 2016

of the minor criteria as follow: pleuritic chest pains, dyspnea, pulmonary consolidation by physical examination, and white blood cell count of > 12000 cells/µl or pulmonary infiltrate seen on a chest radiograph.

Exclusion criteria were patient who refused treatment, diagnosed with tuberculosis or human immunodeficiency virus (HIV) infection and those with age of < 5 years.

This study was approved by Institutional Review Board (IRB) Committee of Zagazig Faculty of Medicine. An informed written consent was taken from each patient or his/her guardian after being informed about the nature as well as the purpose of the study. Participants' data was kept confidential.

Clinical samples collection

Lower respiratory tract specimens were collected using standard Microbiologic procedures and stored at -20°C until processed [16].

Environmental water samples collection

One litre water samples were collected from 25 water outlets in chest department, emergency ICU and surgery ICU using standard Microbiologic procedures and filtered through a membrane filter (0.45 mm pore size, Millipore corporation, Bedford, USA) in a stainless-steel filter holder with a water aspirator. After filtration, membranes were placed into 5ml of sterile water and scraped to remove bacteria. The concentrate was stored at -20 °C until used [17].

DNA extraction

Bacterial DNA was extracted directly from both clinical and water samples using QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France). Procedures followed the instructions of the manufacturer.

Real time PCR of 16s r-RNA gene

Legionella genus was detected in DNA extracts by real time PCR amplification of 16s r-RNA gene using Primerdesign™ genesig® Kit for *Legionella* (all species) (PrimerDesign, UK) whose anchor nucleotide information is; accession number: DQ123630, anchor nucleotide: 71 and context length: 157bp. Procedures were done according to the manufacturer's instructions. For the negative control reaction; RNAse/DNAse free water (supplied in the kit) was used. The following amounts were added to each reaction; ten µL of RT-PCR Master mix, one µL of the primer/probe, four µL of distilled water free of RNAse/DNAse and five µL of the DNA extract. The device used was Real-time PCR instrument Stratagene Mx 3005P (Agilent technologies, USA). The protocol used for amplification was enzyme activation at 95°C for ten minutes followed by 50 cycles of denaturation at 95°C for ten seconds and data collection at 60°C for 60 seconds. The results of the real-time PCR were expressed as threshold cycle (CT) values corresponding to the cycle at which PCR enters the exponential phase. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative.

Clinical data and risk factors determination

Using statistical analyses listed below, the following comparisons were done:

Signs and symptoms of cases of community acquired legionellosis versus those among cases of CAP due to other causes.

Risk factors for community acquired legionellosis versus those among cases of CAP due to other causes.

Clinical outcome for community acquired legionellosis versus those among cases of CAP due to other causes.

Risk factors for hospital acquired legionellosis versus those among cases of HAP due to other causes.

Statistical Analysis

Collected data were computerized and statistically analyzed using SPSS program (Statistical Package for Social Science) version 18.0. Qualitative data were represented as frequencies and relative

percentages. Chi square test was used to calculate difference between qualitative variables. Quantitative data were expressed as mean ± SD (Standard deviation). Logistic regression analysis was used to illuminate the interrelation within and between significant predictors for specific variable. The level of significance for all statistical tests was determined. The threshold of significance is fixed at 5% level (*P*-value); $P > 0.05$ indicates non-significant results, *P*-value of <0.05 indicates significant results.

Results

Real time PCR results revealed that out of the 50 patients with CAP, eight patients' samples were positive for *Legionella* genus, whereas 10 out of 50 of patients with HAP were positive (20%); six of them were in emergency ICU (27.3%), while four patients were in surgery ICU (14.3%) **(Figure 1).** Real time PCR amplification plot is shown in **(Figure 2).**

Characters of cases of community acquired LD compared to CAP due to other causes are shown in **table 1.** Cases with *Legionella* CAP were more likely to be smoker and diabetic males of old age who are immune- suppressed with COPD, and/or malignancy. They were more likely to present with fever, non-productive cough, GIT and neurological manifestations. Cases with *Legionella* CAP were more likely to require ICU admission. When binary logistic regression analysis was made, smoking, male gender, old age, diabetes mellitus and COPD remained significantly associated with *Legionella* CAP, **table 2.**

When risk factors for hospital acquired legionellosis were compared with HAP due to other causes; hospitalization for more than 10 days, stroke or head trauma and heart disease were significantly associated with hospital- acquired LD, **table 3.** Binary logistic regression analysis for risk factors of hospital acquired LD revealed that hospitalization for more than 10 days, stroke or head trauma are the significant risk factors, **table 4.**

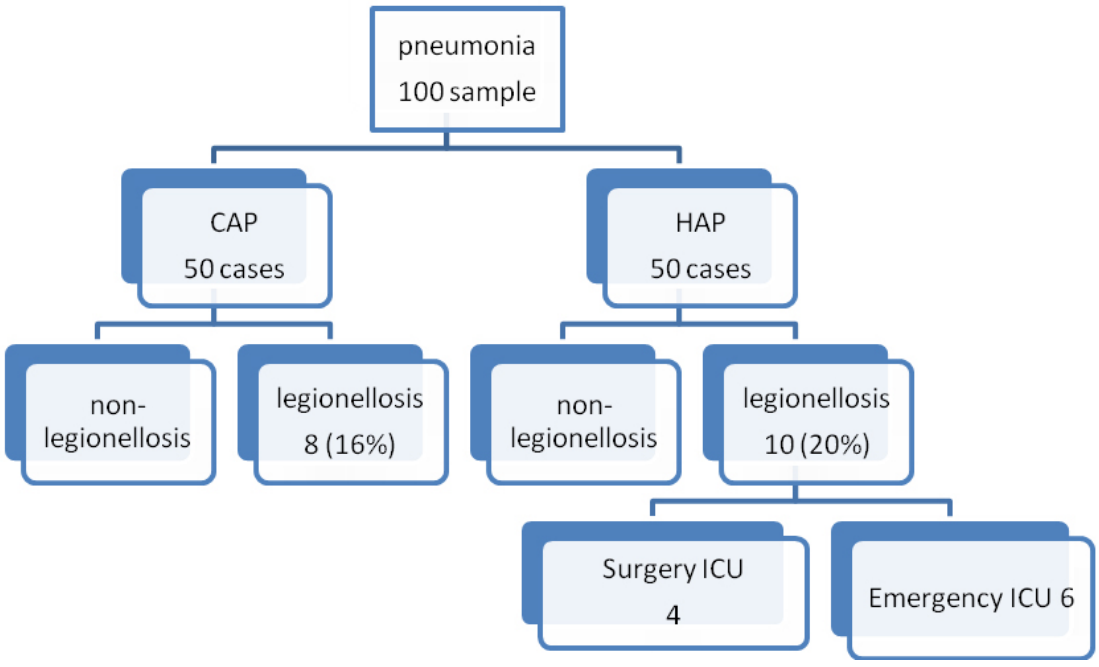

Figure 1. Distribution of cases of CAP and HAP.

Received on 14 May 2016, Accepted on 2 June 2016

ISSN 2174-9094
2016
Vol. 6 No. 2:1
doi: 10.3823/786

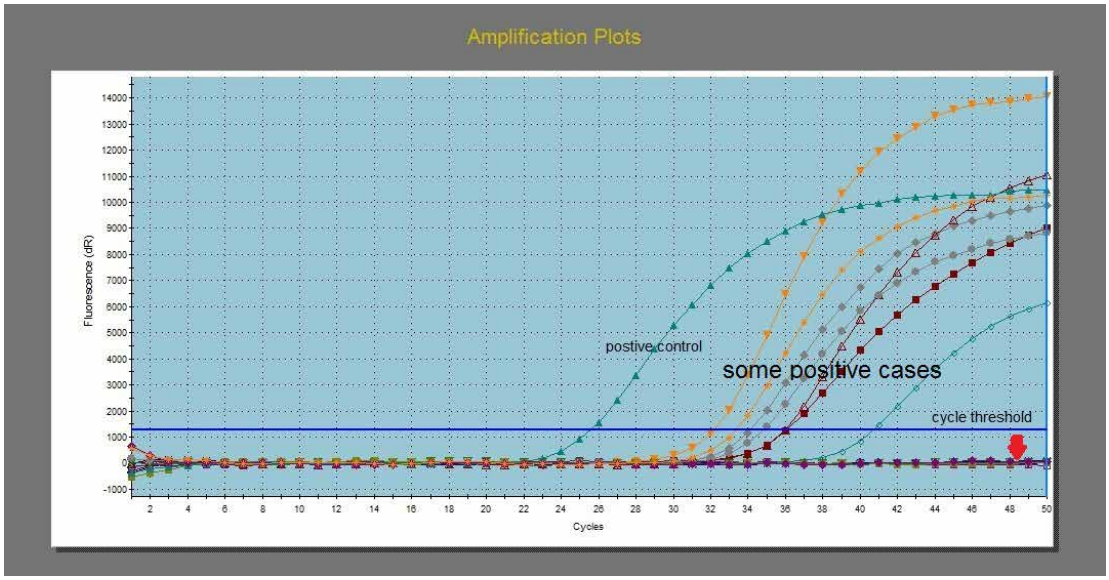

Figure 2. Amplification plot of real time PCR. The results of the real-time PCR are expressed as threshold cycle (CT) values corresponding to the cycle at which PCR enters the exponential phase. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative. The horizontal axis represents 50 cycles of amplification while the vertical axis represents fluorescent signal. This figure shows positive control (CT 25.6), a number of positive cases (variable CTs), negative control (no CT) and a number of negative cases (no CT) (negative control and negative cases are overlapping the horizontal axis of the plot marked by the red arrow)

Ten out of the 25 water samples tested were positive for *Legionella* genus; 7 were from chest department. The remaining three were from emergency ICU.

Discussion

Studies about LD are rare in the developing world, and the problem of *Legionella* is undoubtedly underestimated [4]. Although *L. pneumophila* serogroup 1 is the most common human pathogen of the genus *Legionella*; it is possible that, under favourable situations, the majority of other species and serotypes are also incriminated in Legionnaires' disease due to their competency for cellular invasion and intracellular proliferation [18, 2].

Investigated patients suffering from CAP and needing hospitalization were admitted to the chest department. HAP patients were recruited from emergency and surgery departments because both have the biggest ICUs in our hospital. Moreover, there were complaints of the high frequency of HAP in these two departments and we have no previous information about occurrence of *Legionella* in our hospital.

The diagnosis of *Legionella* infection can be done by a number of investigation arrays. Bacterial culture is considered the most specific means, but it is associated with long time, low sensitivity and technical difficulty. Direct Fluorescent-Antibody (DFA) test is much more rapid than culture, but it has poor sensitivity. Radioimmunoassay, enzyme immunoassay, and latex agglutination can be used for detection of only *L. pneumophila* (serogroup 1) in urine with a moderate sensitivity. Serologic methods are extremely sensitive, but these require long time to detect seroconversion in patients. Analyzes based on molecular diagnostics have emerged recently. Conventional molecular techniques are labor intensive and often need at least 1 day to be achieved. Additionally, the obligatory manipulation

Table 1. Characters of community acquired legionellosis compared to CAP due to other causes.

Characters		CA legionellosis[a] No (%) 8 (100%)	Other causes of CAP[b] No (%) 42 (100%)	P value[c]
Male sex		6 (75%)	14 (33.3%)	0.04
Age (Mean ± Standard Deviation)		49.7 ± 10.3	47.5 ± 1.7	<0.04
Underling medical condition	Diabetes mellitus	3 (37.5%)	3 (7.1%)	0.02
	Hypertension	2 (25%)	6 (14.3%)	0.86
	COPD[d]	2 (25%)	1 (2.4%)	0.01
	Smoking	4 (50%)	6 (14.3%)	0.03
	Immune suppression	1 (12.5%)	0 (0%)	0.02
	Malignancy	1(12.5%)	0 (0%)	0.02
	Stroke or head trauma	0 (0%)	3 (7.1%)	0.44
	Heart disease	1 (12.5%)	9 (21.4%)	0.92
	Liver disease	0 (0%)	6 (14.3%)	0.59
	Kidney disease	1 (12.5%)	3 (7.1%)	0.84
Signs and symptoms	Fever>38	8 (100%)	26 (61.9%)	0.03
	Cough	5 (62.5%)	29 (69%)	0.72
	Sputum	1(12.5%)	22 (52.4%)	0.04
	Dyspnea	4 (50%)	24 (57%)	0.71
	Chest pain	1(12.5%)	4 (9.5%)	0.78
	GIT manifestation[e]	3 (37.5%)	4 (9.5%)	0.03
	Neur. manifestations[f]	2 (25%)	1 (2.4%)	0.01
Clinical outcome	ICU stay	6 (75%)	14 (33.3%)	0.04
	Ventilator support	5 (62.5%)	17 (40.5%)	0.11

a community acquired legionellosis
b other causes of community acquired pneumonia
c P-value of <0.05 indicates significant results, P-value of <0.01 indicates highly significant results
d Chronic obstructive pulmonary disease
e Gastrointestinal tract manifestations
f Neurological manifestations

of post-amplification products escalates the hazards of carryover contamination and consequently false positivity. Currently, using real-time PCR instrumentation allows amplification and identification in a single sealed cuvette. This method eliminates the necessity for additional manipulation of the specimens, significantly diminishes turnaround time and reduces the risk of cross-contamination in samples. Recent reports confirmed that real-time PCR methods are attractive substitutions to conventional PCR

Received on 14 May 2016, Accepted on 2 June 2016

Table 2. Binary logistic regression analysis for significant predictors of community acquired legionellosis

Variable		B[a]	S.E[b]	Wald[c]	Sig.[d]	ExpB[e]	95.0% C.I.[f]	
							Lower	Upper
Risk factors	Age	2.135	1.039	4.220	0.040	8.456	1.103	64.818
	Male sex	2.295	0.474	23.449	0.000	9.925	3.920	25.127
	Hypertension	-0.939	1.009	0.865	0.352	0.391	0.054	2.827
	DM[g]	-2.647	0.446	35.215	0.000	0.071	0.030	0.170
	COPD[h]	1.858	0.488	3.095	0.039	2.360	0.907	6.140
	Smoking	1.329	0.412	10.416	0.001	3.778	1.685	8.469
	Immune suppression	-0.684	0.421	2.637	0.104	0.505	0.221	1.152
Signs and symptoms	Fever	0.81	0.43	0.53	0.61	0.79	0.354	1.791
	Sputum	1.82	0.56	7.12	0.04	3.45	2.12	7.14
	GIT manif.[i]	3.12	0.54	11.22	<0.001	9.56	4.56	16.34
	Neur. manif.[j]	2.11	0.49	8.09	0.02	4.35	2.56	11.55

a Regression coefficients
b Standard error around the coefficient for the constant
c Wald chi-square test statistic
d P-value for Wald test, <0.05 indicates significant results, <0.01 indicates highly significant results
e Expected Beta
f Confidence Interval
g Diabetes mellitus
h Chronic obstructive pulmonary disease
i Gastrointestinal tract manifestation
j Neurological manifestations

techniques [19]. Therefore, real time PCR was used for the identification of the genus *Legionella* in this study.

The gene amplified in the current work; 16S rRNA gene is exceptionally suited as a target for identification of the genus *Legionella* since it is highly conserved and exists in several copies per genome and thus allows a high sensitivity of the PCR in both clinical and environmental water samples. However, the variation in the 16S rRNA is not large enough to resolve strains or serogroups within a genus. For species identification, other methods need to be applied [20, 21]. Since *Legionella* is not a part of the normal human flora, quantification was not indicated for clinical samples and presence or absence of the organism could be considered satisfactory [22].

The rate of isolation of *Legionella* genus (16%) among our CAP patients is shown in figure 1. It is higher than that of an earlier Egyptian study (5% with CAP) [13]. The lower incidence of the previous study could be attributed to their method of investigation which has focused only on *L. pneumophila.*

Few countries of the world consider LD a notifiable disease, and incidence of *L. pneumophila* and other species can be obtained, whereas, most other countries have rare data because of lack of diagnostics and surveillance systems. It is worth mentioning that the worldwide occurrence of Legionnaires' disease is challenging to measure and attention should be taken in explanation of the surveillance figures [10].

Similar to many previous reports, our study showed that community acquired legionellosis was more common in old- age and smokers male patients **(Tables 1 & 2).** Smoking as a risk factor for legionellosis was found in previous studies as the following, 63%, 25% and 15.4%, respectively [23-

Table 3. Risk factors for hospital acquired legionellosis compared with HAP due to other causes.

Risk factor	HA legionellosis[a] No (%) 10 (100%)	Other causes of HAP[b] No (%) 40 (100%)	P value[c]
Age (M[d] ± SD[e])	37.4 ± 12.7	34.5 ± 10.3	0.54
Male sex	8 (80%)	28 (70%)	0.66
Diabetes mellitus	1 (10%)	14 (35%)	0.24
Hypertension	2 (20%)	8 (20%)	0.95
COPD[f]	1 (10%)	5 (12.5%)	0.83
Smoking	5 (50%)	13 (32.5%)	0.57
Hospitalization ≥ 10 ds	9 (90%)	12 (30%)	<0.001
Intubation	9 (90%)	31 (77.5%)	0.66
Immune suppression	1 (10%)	1 (2.5%)	0.86
Malignancy	1 (10%)	13 (32.5%)	0.31
Stroke or head trauma	6 (60%)	10 (25%)	0.03
Heart disease	4 (40%)	2 (5%)	0.002
Liver disease	1 (10%)	3 (7.5%)	0.79
Kidney disease	1 (10%)	1 (2.5%)	0.86

a Hospital acquired legionellosis
b Other causes of hospital acquired pneumonia
c P-value of <0.05 indicates significant results, P-value of <0.01 indicates highly significant results
d Mean
e Standard deviation
f Chronic obstructive pulmonary disease

25]. Smoking raises the risk of legionellosis by 121% for each pack of cigarettes used up daily. This increased risk is due to the difficulty in eliminating the microorganism from the bronchial tree, due to the worsening of the respiratory mucosa and weakened cilia caused by tobacco smoking that facilitates entry into and subsequent invasion of the alveolar macrophages, depending on the individual's immune rank [26]. In the current study, diabetes was a significant risk factor for legionellosis **(Table 1 & 2).** This result is in agreement with former studies which showed that diabetes was detected in 23-41% of patients [23-25]. Diabetic patients were found to be at increased risk of legionellosis due to impaired cell mediated immunity [27]. This study found that COPD was another risk factor **(Table 1 & 2)** which agrees with the study of Maniwa *et al.* who reported that 21% of patients with LD suffered from COPD [23], while other authors reported lower rates of COPD occurrence [25]. Chronic renal disease, having a previous stroke, hypertension and malignancy were not significant risk factors in our study as compared to other investigations [23, 25]. The diversity of results may be related to variable environmental exposure or due to variable susceptibility of different populations [21].

In this study, clinical presentations of patients with community acquired legionellosis have similar features to other reported studies **(Table 1 & 2).** In addition, GIT and neurologic symptoms were previously reported in LD, with a range of 20% to 50% [23-25, 27-30], whereas our finding showed that 75% of patients with community-acquired legionellosis were admitted to ICU, and this factor is comparable with other studies [25]. This result can be attributed to old age and underlying co-morbidity of patients with *legionella* pneumonia which causes severe disease [31].

Received on 14 May 2016, Accepted on 2 June 2016

Table 4. Binary logestic regression analysis for significant predictors of hospital acquired legionellosis:

Variable	B[a]	S.E[b]	Wald[c]	Sig.[d]	ExpB[e]	95.0%	C.I.[f]
						Lower	Upper
Hospitalization ≥ 10 ds	-0.967	0.345	7.849	0.005	2.379	1.109	5.105
Stroke or head trauma	-1.104	0.386	8.173	0.004	2.420	1.048	5.591
Heart disease	-0.236	0.411	0.329	0.566	0.792	0.451	0.984

a Regression coefficients
b Standard error around the coefficient for the constant
c Wald chi-square test statistic
d P-value for Wald test, <0.05 indicates significant results, <0.01 indicates highly significant results
e Expected Beta
f Confidence Interval

The current incidence of hospital-acquired LD **(figure 1)** falls within previous reported rates which were reported between one to 40% of cases [1, 32]. This wide range of occurrence stated by those studies probably reflects the difficulty of accurate estimation of such cases. Centres for Disease Control and Prevention (CDC) estimate that less than ten percent of hospital-acquired LD cases are actually reported [33].

It was not possible to compare between clinical manifestations of HA- *Legionella* patients versus HA non-*Legionella* cases due to difficulty in collecting data particularly in ICU settings [31]. Hospitalization for more than 10 days was a major risk factor for hospital acquired legionellosis **(Table 3 & 4)** [34]. Stroke or head trauma was also a significant risk factor, a finding that could be attributed to the impaired conscious level which is linked with higher frequency of aspiration.

Fortunately, the early empirical antibiotics used for treatment of pneumonia patients in our hospital according to the guidelines of the American Thoracic Society and the Infectious Diseases Society of America for CAP [fluoroquinolone or (beta- lactam plus macrolide) [35], and HAP (beta- lactam plus fluoroquinolone) [15], included drugs that are effective against *Legionella* infection.

Nosocomial *Legionella* infection can occur through aerosol formation or aspiration. The water samples collected in this work were obtained from potable outlets (tap water). The presence of *Legionella* in the hospital potable water has been suspected as the only certain risk factor for contracting hospital acquired LD [11, 12]. Moreover, in our hospital, all water used for general care of patients is the potable water, particularly for cleaning and filling humidifiers, respiratory devices, nebulizers, bathing, toilet flushing and nasogastric and endotracheal intubation. Therefore, if tap water is contaminated, *Legionella* can easily spread and cause infection in susceptible hosts [36, 37].

This study is the first work investigating the presence of *Legionella* genus in the water of our hospital. It has been concluded that *L. pneumophila* and *Legionella* species other than *pneumophila* may be isolated together or alone from water [38, 39], since presence of *L. anisa* may mask water contamination by *L. pneumophila* [40]. Knowing that our surgery ICU is a new building located away from the other 2 departments can provide an answer to the question, why water sources in the surgery ICU are free of *Legionella* genus. Based on our findings and on the fact that there is no person-to person transmission of *Legionella*, we assume that the positive cases in the surgery ICU may have acquired the infection during their hospitalization in the pre- operative period, or during the operation itself. Therefore, unless sound infection control policies and procedures are

implemented, admission of patients infected with *Legionella* from other hospital areas to the new ICU may enhance spreading of *legionella* infection by contamination of diagnostic and therapeutic equipment through Legionella- carrying aerosol.

Conclusions: This study found that old –age, smoker– males having CAP with an underlying diabetes mellitus and/or COPD and presenting with non-productive cough, and GIT and neurological manifestation should be considered for *Legionella* infection. Whereas, patients hospitalized for more than 10 days, and having a stroke or head trauma who acquired HAP should be suspected for *Legionella* infection. Rigorous infection control measures are required to keep the water sources in the surgery ICU clean to prevent any contamination with *legionella*.

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 Received on 14 May 2016, Accepted on 2 June 2016

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